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CORRELATION OF REAL-TIME CATECHOLAMINE RELEASE AND CYTOSOLIC CA^{2+} AT SINGLE BOVINE CHROMAFFIN CELLS

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Previous investigations of the role of Ca^{2+} in stimulus-secretion coupling have been undertaken in populations of adrenal chromaffin cells. In the present study, the simultaneous detection of intracellular Ca^{2+}, with the fluorescent probe fura-2, and catecholamine release, using a carbon-fiber microelectrode, are examined at single chromaffin cells in culture. Results from classic depolarizing stimuli, high potassium (30-140 mM) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) (3-50 μM), show a dependence of peak cytosolic Ca^{2+} concentration and catecholamine release on secretagogue concentration. Catecholamine release induced by transient high K^+ stimulation increases logarithmically with K^+ concentration. Continuous exposure to veratridine (50 μM) induces oscillations in intracellular Ca^{2+}, and at higher concentrations (100 μM) concomitant fluctuation of cytosolic Ca^{2+} and catecholamine secretion. Mobilization of both caffeine- and IP_3-sensitive intracellular Ca^{2+} stores is found to elicit secretion with or without extracellular Ca^{2+}. Caffeine-sensitive intracellular Ca^{2+} stores can be depleted, refilled, and cause exocytosis in medium without Ca^{2+}. Single-cell measurement of exocytosis and the increase in cytosolic Ca^{2+} induced by bradykinin-activated intracellular stores reveal cell-to-cell variability in exocytotic responses which is masked in populations of cells. Taken together, these results show that exocytosis of catecholamines can be induced by an increase in cytosolic Ca^{2+} either as a result of transmembrane entry or by release of internal stores.

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Correlation of Real-Time Catecholamine Release and Cytosolic Ca^{2+} at Single Bovine Chromaffin Cells

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SUMMARY

Previous investigations of the role of Ca^{2+} in stimulus-secretion coupling have been undertaken in populations of adrenal chromaffin cells.[1-4] In the present study, the simultaneous detection of intracellular Ca^{2+}, with the fluorescent probe fura-2, and catecholamine release, using a carbon-fiber microelectrode, are examined at single chromaffin cells in culture. Results from classic depolarizing stimuli, high potassium (30-140 mM) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) (3-50 μM), show a dependence of peak cytosolic Ca^{2+} concentration and catecholamine release on secretagogue concentration. Catecholamine release induced by transient high K^+ stimulation increases logarithmically with K^+ concentration. Continuous exposure to veratridine (50 μM) induces oscillations in intracellular Ca^{2+}, and at higher concentrations (100 μM) concomitant fluctuation of cytosolic Ca^{2+} and catecholamine secretion. Mobilization of both caffeine- and IP_3-sensitive intracellular Ca^{2+} stores is found to elicit secretion with or without extracellular Ca^{2+}. Caffeine-sensitive intracellular Ca^{2+} stores can be depleted, refilled, and cause exocytosis in medium without Ca^{2+}. Single-cell measurement of exocytosis and the increase in cytosolic Ca^{2+} induced by bradykinin-activated intracellular stores reveal cell-to-cell variability in exocytotic responses which is masked in populations of cells. Taken together, these results show that exocytosis of catecholamines can be induced by an increase in cytosolic Ca^{2+} either as a result of transmembrane entry or by release of internal stores.

Secretion of cellular substances often occurs by exocytosis, a process which involves the fusion of intracellular vesicles containing hormones and/or neurotransmitters with the plasma membrane.[5,6] The bovine adrenal chromaffin cell releases the catecholamine hormones, epinephrine and norepinephrine, in this way. Although the details of the exocytotic mechanism remain unclear at the molecular level, calcium influx is known to be an essential trigger for the exocytotic process in adrenal chromaffin and other cells.[7,8] Cytosolic free Ca^{2+} can be increased in two ways: depolarizing stimuli can increase cytosolic Ca^{2+} via influx of extracellular Ca^{2+} through calcium channels[9,10] or, alternatively, release from intracellular Ca^{2+} stores can increase cytosolic free Ca^{2+}.[11,12] The role of intracellular Ca^{2+} stores in the exocytotic process remains controversial.[3,13,14] One reason for this controversy is that measurements of catecholamine release and Ca^{2+} entry are often made in separate cell preparations. Measurements in populations may conceal certain effects due to heterogeneity within cell populations, as shown recently with Ca^{2+}/fura-2 measurements.[15] Thus, to further define the role of Ca^{2+} in exocytosis, it is necessary that the elevation of cytosolic free Ca^{2+} and concomitant secretion be quantitated and simultaneously correlated at single cells.

Measurements of intracellular Ca^{2+} in single cells is possible with fluorescent probes,[16,17] but until recently measurements of secretion were normally made in populations of cells.[13,18] Release from single cells has been indirectly monitored by examining the effects on cocultured cells[19] or by measurements of changes in whole cell capacitance.[20] The direct measurement of secretion from single cells with carbon-fiber microelectrodes has now been achieved, enabling much higher resolution of individual vesicular secretion events.[21-24] The present study employs fura-2 fluorescence[16] as a probe of cytosolic free Ca^{2+} and a carbon-fiber microelectrode, placed adjacent to the cell, to monitor released catecholamine[21,25] resolved at the individual vesicular level. The fluorescent measurements give a measure of average changes in cytosolic free Ca^{2+} throughout the cell, whereas the electrochemical signals record the individual exocytotic events which occur at the region of the cell surface directly beneath the sensor tip.[24]

The chromaffin cell is an excellent system to probe the role of calcium in stimulus-secretion coupling because it has been shown to undergo calcium-dependent catecholamine release[1,3,26] and has been extensively used a model for neurosecretion.[9,27] Many previous studies of the relationship of these events have been undertaken in chromaffin cell populations[1-4,28] and perfused adrenal glands.[29]

The results presented in this paper show that catecholamine release, resolved at the level of single cultured cells, correlates well with cytosolic free Ca^{2+} levels when classical depolarizing secretagogues which cause Ca^{2+} influx through Ca^{2+} channels are employed. In contrast, agents which liberate Ca^{2+} from caffeine-sensitive or IP_3-sensitive stores[3,14] show more variable responses from cell to cell. These agents can induce exocytotic secretion in the absence of extracellular Ca^{2+} in some cells. Other cells do not exhibit secretion even in the presence of extracellular Ca^{2+} when cytosolic free Ca^{2+} is elevated by release of an intracellular store. The heterogeneity revealed in these studies indicates that interpretation of the exocytotic mechanism requires single-cell measurements of cytosolic free Ca^{2+} and exocytotic release.

EXPERIMENTAL PROCEDURES

Chromaffin cells and solutions--Primary cultures of bovine adrenal medullary cells were prepared from fresh tissue,[25] enriched in epinephrine using a single-step Renografin gradient,[30] and plated on glass coverslips (Carolina Biological Supply, Burlington, NC) at a density of 6 x 10^5 cells per 35-mm diameter plate. All experiments were performed at room temperature between days 3 and 8 of culture. For all experiments the culture medium was replaced with Krebs-Ringer buffer containing (in mM) 145 NaCl, 5 KCl, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, and 20 HEPES. Either 2 mM $CaCl_2$ or 0.2 mM EGTA (to give a free extracellular Ca^{2+} level < 10^{-8} M)[31] was added to achieve the desired Ca^{2+} content, and all solutions were adjusted to pH 7.4 with NaOH. All experiments were performed on the stage of an inverted microscope (Axiovert 35, Zeiss, Thornwood, NY). When veratridine was employed, a small volume of concentrated stock solution was added to the plate at the indicated time. Other secretagogues were locally applied for 3-5 s every 2 min via pressure ejection from glass micropipettes using a Picospritzer (General Valve Corp., Fairfield, NJ). When potassium was used as a secretagogue, the concentration of NaCl in the pipette was reduced to maintain osmolarity.

Electrochemical measurement of secretion--Carbon-fiber microelectrodes were prepared by sealing individual fibers (5-µm radius, Thornell P-55, Amoco Corp., Greenville, SC) into glass pipettes with epoxy (Epon 828 Resin and *m*-phenylenediamine hardener, Miller-Stephenson, Danbury, CT). Electrodes were polished at a 45° angle on a micropipette beveller (Model BV-10, Sutter Instruments, Novato, CA) and then soaked in 2-propanol for at least 15 min before use.[32] Calibrations were performed using a flow-injection apparatus with 50 µM epinephrine.[24] Amperometric measurements ($E_{applied}$ = +650 mV vs. sodium-saturated calomel electrode) employed an EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN) in two electrode mode. The carbon-fiber working electrodes were positioned 1 µm away from the cell with a piezoelectric driver (PCS-1000 Patch Clamp Manipulator, Burleigh Instruments, Fishers, NY) as

previously described.[22] This arrangement has been shown to measure secretion from a region extending 2 μm beyond the carbon-fiber perimeter.[24] Amperometric electrode responses were low pass filtered at 16.67 kHz, digitized using a PCM-2 A/D VCR Adaptor (Medical Systems Corp., Greenvale, NY), and recorded on 1/2" videotape. For analysis, the data was low-pass filtered at 25 Hz (Krohn-Hite 3750, Avon, MA), digitized at 20 ms/pt using a NIC-310 oscilloscope (Nicolet Instrument Corp., Madison, WI) and imported into a personal computer. Locally written software was used to determine the area under the current versus time traces for 60 s following secretagogue delivery. In this way the quantity of catecholamine released under the electrode from a single exposure to a secretagogue was evaluated. Charge can be related to the number of moles of catecholamine detected with Faraday's law:

$$Q = n F m \qquad \text{(Eq. 1)}$$

where Q = area under the current versus time trace for 60 s following secretagogue delivery (charge, in coulombs), F = Faraday's constant (96,485 coulombs/equivalent), n = number of electrons passed in reaction per mol (n = 2 for catecholamine) and m = total number of moles of catecholamine detected by electrode.

Fura-2 Calcium Measurements--Chromaffin cells were incubated in Krebs-Ringer buffer containing 1 μM fura-2 AM (stock solution dissolved in 20% Pluronic F-127 in DMSO), 0.1% bovine serum albumin, and 2 mM Ca^{2+} for 30 min at room temperature. Culture plates were rinsed twice and refilled with buffer with the desired Ca^{2+} content. Single cells were selected for fluorescence measurement using a 43 μm pinhole aperture with the EMPIX Photometer System (Mississaugua, Canada). Cells were alternately excited at 340 nm and 380 nm and light was collected through a 40 x oil-immersion objective (Fluar 40 X, Zeiss, Thornwood, NY).[31] To reduce photobleaching, a 0.5 neutral density filter was placed between the excitation source and sample, and fluorescence was sampled every 250 ms. Since the presence of the microelectrode

induced considerable autofluorescence and some reflectance, it was necessary to correct the measured fluorescence intensities. Data were not corrected for cell autofluorescence because it could not be measured at the specific cells used. Autofluorescence is significant, however was found to vary from cell-to-cell, and its contribution to the measured signal leads to an underestimate of cytosolic Ca^{2+} concentrations. The corrected fluorescence values were ratioed (F_{340}/F_{380}) and estimates of intracellular Ca^{2+} concentration were calculated using a previously published method.[16] Errors for fluorescent and electrochemical measurement are given as standard errors of the mean.

Chemicals--Culture medium, Dulbecco's modified Eagle's/Ham's F-12 medium (DMEM/F12), was obtained from Gibco Laboratories (Grand Island, NY). Collagenase (Type I) for digestion of gland tissue was obtained from Worthington Biochemical (Freehold, NJ). Renografin-60 was purchased from Squibb Diagnostics (New Brunswick, NJ). Fura-2 AM, free acid, and Pluronic-F127 were obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma (St. Louis, MO), and solutions were prepared with doubly distilled water.

RESULTS

Effect of repetitive high K^+ stimulations--Figure 1 shows the Ca^{2+} response (upper trace) and catecholamine release (lower trace) induced by 3-s delivery of 60 mM K^+ repeated at 2 min intervals. A steady-state, low cytosolic free Ca^{2+} concentration and no exocytotic events were exhibited at cells before the stimuli were applied. Upon application of K^+, the fluorescent ratio from fura-2 rose rapidly and current spikes from the exocytotic release of catecholamine were observed in a parallel time course. After the cytosolic free Ca^{2+} reached a maximal concentration, a slower decline in the fluorescent ratio commenced. At this time, the frequency of exocytotic spikes decreased until cytosolic free Ca^{2+} fell below the threshold required to maintain exocytosis and catecholamine spikes ceased. The shift in baseline observed after stimulation in the electrochemical traces is due to the overlap of many catecholamine spikes.

Figure 2 shows both Ca^{2+} influx and catecholamine release at a single cell exposed to various concentrations of K^+. The mean total charge due to catecholamine release detected from a single exposure to 140 mM K^+ was 385 ± 36 pC (corresponding to 2.0 ± 0.19 femtomoles of catecholamine) and the mean apparent maximal cytosolic Ca^{2+} was 330 ± 23 nM. The maximal free Ca^{2+} and catecholamine secretion were found to be dose dependent as application of 30 mM K^+ elicited 39 ± 9.6% of the mean apparent maximal cytosolic Ca^{2+} response and only 4.2 ± 9.4% of the release of that from 140 mM K^+. Transient delivery of 20 mM or 10 mM K^+, by pressure ejection, did not elicit detectable secretion or changes in cytosolic free Ca^{2+} from basal level. Pooled results of the maximal cytosolic Ca^{2+} and catecholamine release from 6 cells are plotted versus log K^+ in figure 3.

Concentration dependence of DMPP induced changes--Figure 4 shows the concentration dependence of cytosolic free Ca^{2+} and catecholamine secretion for a single cell exposed to DMPP, a nicotinic agonist known to trigger Ca^{2+} influx. As with high K^+, increasing the secretagogue concentration increased resultant cytosolic free Ca^{2+} and catecholamine

responses. Delivery of 3 μM DMPP resulted in 48 ± 7.9% of the maximal apparent cytosolic Ca^{2+} response and 12 ± 9.6% of the catecholamine release found with 50 μM DMPP (232 ± 17 nM and 535 ± 50 pC (2.8 ± 0.26 femtomoles of catecholamine), respectively). The durations of both cytosolic free Ca^{2+} elevation and catecholamine release from DMPP are longer than those for K^+ (45-80 s versus 25-60 s).

Responses to veratridine--Preliminary experiments showed that transient (3-s) exposure of cells to 100 μM veratridine did not effect cytosolic free Ca^{2+} or induce catecholamine release. Therefore veratridine, an agent that activates plasma membrane Na^+ channels,[18,33] was added to the entire culture plate at the time indicated on the trace to give the desired concentration. Figure 5 shows the results induced by veratridine following a 3-s application of 60 mM K^+ to confirm viability of the cell. Veratridine (50 μM) was found to cause oscillations in the internal Ca^{2+} concentration (from apparent basal levels of 40 nM to approximately 85 nM) which were insufficient to cause significant catecholamine release (n = 7 cells). Oscillations of free Ca^{2+} were suppressed and intracellular Ca^{2+} were returned to basal levels by transient application of 10 μM tetrodotoxin (TTX) (data not shown). Upon increasing the veratridine concentration to 100 μM, Ca^{2+} oscillations increased in magnitude and frequency and catecholamine release began to parallel this pattern in 5 of the 8 cells (Fig 5, inset). Two cells required 200 μM to cause release to mimic the cytosolic free Ca^{2+} transients. After about 90 s of simultaneous oscillatory behavior, the cell cytosolic free Ca^{2+} and catecholamine release reached a sustained elevated state. The final state of high activity continued for several minutes. In one cell sequentially exposed to 50, 100, and 150 μM veratridine, an increase in the frequency of Ca^{2+} oscillations was noted with increased veratridine but exocytotic release never occurred. In this cell, the maximal cytosolic free Ca^{2+} concentrations during the oscillations remained low.

Effects of caffeine delivery in the presence and absence of extracellular Ca^{2+}--Caffeine pressure ejected in the presence of extracellular Ca^{2+} was found to induce a rise in cytosolic free Ca^{2+} and exocytotic catecholamine release in single cells (Figure 6A). Regardless of the order of

delivery, 40 mM caffeine always elicited larger fura-2/Ca^{2+} response and catecholamine release than did 10 mM. The cytosolic free Ca^{2+} responses induced by caffeine in Ca^{2+}-containing medium consisted of two phases: a rapid transient, attributed to Ca^{2+} expulsion from internal stores, and a longer-lasting plateau due to influx of extracellular Ca^{2+}.[11] The second phase was not apparent in Ca^{2+}-free medium. Results from repetitive stimulations of caffeine remained consistent, with only slightly diminished peak cytosolic free Ca^{2+} values in 5 cells.

In the absence of extracellular Ca^{2+} (0.2 mM EGTA), the first application of 10 mM or 40 mM caffeine always elicited a fast increase in cytosolic free Ca^{2+} and exocytotic release (Figure 6B). Subsequent stimulations of either concentration resulted in a smaller peak cytosolic free Ca^{2+} concentration and very little or no release of catecholamine (n = 7 cells). A third stimulation with caffeine could not elicit any cytosolic free Ca^{2+} rise or catecholamine release. A 3-s application of 10 μM DMPP with 2 mM Ca^{2+} after the caffeine applications verified that the exocytotic machinery of the cell was still intact and served to refill caffeine-sensitive stores as subsequent exposure to 10 mM caffeine showed restored cytosolic free Ca^{2+} elevation with simultaneous catecholamine release (Figure 6B).

Heterogeneity of responses from bradykinin application--Individual cells were examined in media with and without Ca^{2+} to examine the effect of bradykinin, which activates B_2-bradykinin receptors present on bovine chromaffin cells and elevates intracellular IP_3 levels which induce a rise in cytosolic free Ca^{2+}.[34-36] Several different patterns in the responses of cytosolic free Ca^{2+} and catecholamine release were obtained in the present study (Figure 7). In medium with 0.2 mM EGTA, 4 out of 15 cells showed both cytosolic free Ca^{2+} increase and robust catecholamine release from delivery of 200 nM bradykinin (Figure 7A). The release in these cases was often longer in duration and larger in quantity (pC) compared to release induced by 60 mM K^+ with 2 mM Ca^{2+} at the same cell. Like caffeine, the cells which did exhibit release only did so for the first exposure indicating that the bradykinin-sensitive internal Ca^{2+} store is also quickly depleted. Of the cells that did not release catecholamine, one had a long-lasting, substantial rise in

cytosolic free Ca^{2+} (Figure 7B). The remaining 10 cells showed neither Ca^{2+} influx nor secretion of catecholamine even though cell viability was substantiated with high K^+ deliveries before and after bradykinin (Figure 7C). When 2 mM Ca^{2+} was present in the extracellular media, 60% of the cells studied behaved as depicted in figure 7A.

DISCUSSION

In this work we have combined fluorescent detection of cytosolic free Ca^{2+} with electrochemical measurement of catecholamine release at the single-cell level to correlate responses to various chemical agents. The methods employed leave the cell membrane unperturbed thus providing a more physiological view of biochemical changes induced in the cell by various secretagogues. The microelectrode reports exocytotic events that occur in the region of the cell membrane directly beneath it.[24] The use of fura-2 AM allows measurement of whole-cell cytosolic Ca^{2+} without the complication of washout of endogenous Ca^{2+} buffers,[37] although, like all chelating fluorescence probes, it may buffer the internal concentration changes that occur.[38-40] The general picture that emerges is that exocytotic secretion in each cell is tightly coupled to an elevation of intracellular Ca^{2+}. However, an increase in intracellular Ca^{2+} is not sufficient to cause release; rather, the intracellular Ca^{2+} concentration must exceed a threshold before release occurs. This is the case whether Ca^{2+} elevation is induced by transmembrane entry or by mobilization of intracellular Ca^{2+} stores.

Transient exposure of a single cell to agents which cause membrane depolarization lead to a concentration dependent increase in cytosolic free Ca^{2+} coupled with catecholamine secretion by exocytosis. Both effects are more short lived with elevated K^+, which causes direct depolarization of the cell membrane, than with DMPP, which acts via the nicotinic receptor. However, in both cases the results are consistent with vesicular release triggered by entry of extracellular Ca^{2+} [41] via voltage-sensitive Ca^{2+} channels.[9] Release and elevation of cytosolic Ca^{2+} remain quite similar with 6 repetitive exposures to K^+, although the maximal free Ca^{2+} concentration decreases slightly with stimulation number, perhaps due to habituation of Ca^{2+} channels.[42]

With both DMPP and K^+ at low concentrations, the relative increases in cytosolic Ca^{2+} are larger than the relative release, consistent with observations made with populations of chromaffin

cells[4,28] and support the finding that a threshold Ca^{2+} concentration is necessary to trigger secretion. This is clearly seen when the normalized responses are plotted versus the log K^+ concentration, which is directly proportional to the degree of membrane depolarization.[43] While secretion linearly increases with membrane depolarization at concentrations above 30 mM, as found for dopamine release from synaptosomes[44], lower concentrations of K^+ do not induce measurable secretion. In contrast, 30 mM K^+ induces a significant rise in cytosolic Ca^{2+} while the two highest concentrations of K^+ tested induce comparable changes in Ca^{2+}. The sigmoidal curve is similar to that found for $^{45}Ca^{2+}$ uptake into brain synaptosomes stimulated with K^+.[43,45] When a logarithmic plot of catecholamine release versus maximal cytosolic free Ca^{2+} is constructed from the pooled data in figure 3, a third-order dependence on Ca^{2+} is found (slope = 3.06, r = 0.964). This supports the view that multiple Ca^{2+} ions act cooperatively at the exocytotic trigger site.[39] Third-order dependence of secretion on intracellular Ca^{2+} also has been observed in synaptosomes,[46] giant squid synapses[47], and via capacitance methods at chromaffin cells.[20,48] Thus, it appears that several aspects of stimulus-secretion coupling are conserved in both endocrine and neuronal systems.

Prolonged opening of Na^+ channels by veratridine[33] causes influx of extracellular Ca^{2+} and may release Ca^{2+} from internal stores, activating Ca^{2+} extrusion mechanisms including the Na^+/Ca^{2+} exchanger.[18,49] The combined effects of this long-lasting activation result in oscillations of cytosolic Ca^{2+} concentration in chromaffin cells. However, as the data show, oscillations of cytosolic Ca^{2+} in bovine chromaffin cells are only accompanied by exocytotic release once a threshold Ca^{2+} value is surpassed. Oscillating release is seen at higher concentrations of veratridine (> 50 μM) that temporally corresponds to the cytosolic Ca^{2+} oscillations. Recently, capacitance measurements have revealed simultaneous exocytosis and Ca^{2+} oscillations in rat gonadotropes.[50] Eventually, the processes which lower cytosolic Ca^{2+} are overwhelmed and both responses remain elevated.

Caffeine induces catecholamine secretion from perfused adrenal glands in both the

presence and absence of extracellular Ca^{2+}.[29] This is in contrast to the depolarizing agents and has lead to the concept that mobilization of internal Ca^{2+} stores can independently induce exocytosis.[29,51] Imaging studies have shown that caffeine-sensitive internal stores of Ca^{2+} are homogeneously distributed throughout the cell, whereas the Ca^{2+} influx induced by depolarizing agents initially occurs at the cellular membrane.[9] Thus, the spatially-averaged values obtained with caffeine more closely reflect the concentration of Ca^{2+} that exists at the release sites.[48,52] Rapid free diffusion of Ca^{2+} in the cell cytosol is unlikely because of the presence of immobile Ca^{2+} buffers.[38-40,53] Since whole-cell measurements of cytosolic Ca^{2+} give an average concentration which will not reflect localization regions of high concentrations, these experiments with caffeine provide a more direct measure of the Ca^{2+} concentration threshold necessary for exocytosis in intact single chromaffin cells. Caffeine, in the absence of extracellular Ca^{2+}, caused a rapid increase of cytosolic Ca^{2+} from its basal level (20 ± 12 nM). (Figure 6B) However, catecholamine release did not occur until cytosolic Ca^{2+} reaches an apparent concentration of 128 ± 27 nM (n=5).[54] This Ca^{2+} threshold concentration is approximately 50 % greater than that found with the preceding exposure to DMPP and Ca^{2+} at the same cell, consistent with the spatial heterogeneity of Ca^{2+} concentration found immediately after delivery of depolarizing agents.[9]

In the absence of extracellular Ca^{2+}, only the initial exposures to caffeine (10 or 40 mM) induced exocytosis of catecholamine because a majority of the contents of the caffeine-sensitive Ca^{2+} stores were initially mobilized and thus remained depleted.[11,55] Restoration of the caffeine-sensitive Ca^{2+} store has been demonstrated by prolonged incubation in Ca^{2+}-containing media,[11,12,56] but this work shows that only a brief elevation (from 3-s, approximately 9 nL, of 10 μM DMPP and 2 mM Ca^{2+}) in cytosolic free Ca^{2+} is required to refill the store sufficiently to induce catecholamine release. The limited duration and quantity of caffeine-induced catecholamine release in Ca^{2+}-free medium may explain the conflicting reports on this topic.[11,13,51,56]

In sharp contrast to caffeine, which was always able to induce catecholamine secretion

by initial release of an internal Ca^{2+} store, bradykinin only could elicit secretion from 27% of cells in the absence of extracellular Ca^{2+}. This difference could be because caffeine-releasable stores contain more free Ca^{2+} than those which are sensitive to bradykinin. Alternatively, since bradykinin releases an IP_3-sensitive store that is near to the nucleus,[36] while caffeine-sensitive stores are more homogeneously distributed,[13,52,55,57] the location of the Ca^{2+} rise may also play a role. The majority of cells exposed to bradykinin showed neither an increase in cytosolic free Ca^{2+} nor secretion of catecholamine, perhaps due to the lack of B_2-bradykinin receptors or a necessary second messenger, or simply that bradykinin-sensitive Ca^{2+} stores were empty. Failure to observe release, even with the sustained increase in cytosolic free Ca^{2+} seen in at one cell, may be because the polarized location of the Ca^{2+} store was at a site distant from the electrode of because the necessary Ca^{2+} threshold was not achieved.

When 2 mM Ca^{2+} was present in the extracellular media, 60% of the cells showed an increase in cytosolic free Ca^{2+} accompanied by release, comparable to previous work.[36] However, the total release from populations of chromaffin cells induced by bradykinin in the presence of external Ca^{2+} was only 20% of that induced by nicotine.[34,36] The present study reveals that this difference in secretion is due in part to the larger number of cells that will secrete in response to nicotine exposure and not necessarily that each single cell secretes more from nicotine stimulations. These results show the possibility of misinterpreting whole population measurements and reveal the benefits of single cell measurements when studying agents with heterogeneous responses in cell populations.

Simultaneous fluorescence detection of cytosolic free Ca^{2+} transients and electrochemical measurement of catecholamine release allows the role of Ca^{2+} in stimulus-secretion coupling to be probed. These studies demonstrate the feasibility of systematic investigations correlating cytosolic free Ca^{2+} with exocytosis at the single-cell level. Results using short- or long-lasting depolarizing stimuli and agents that mobilize Ca^{2+} from caffeine- and IP_3-sensitive internal stores show that different routes to Ca^{2+}elevation usually lead to exocytosis. Further investigations

coupled with molecular biology could elucidate the mechanism of action and specific Ca^{2+} target activated during the short delay between stimulus and vesicular release at the adrenal chromaffin cell.[23]

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54. While corresponding well with single-cell caffeine-induced changes shown by other researchers,[11,55] our apparent maximal cytosolic Ca^{2+} values are lower than traditionally accepted concentrations which culminate in exocytosis.[9,37] One of the reasons for this discrepancy is the difficulty in Ca^{2+} calibration on the single-cell level--*in situ* cell-lysis methods (using digitonin or Triton-X) cannot be performed at single cells because fura-2 leaks away from the measured region and Ca^{2+} ionophores (ionomycin or A23187) do not fully equilibrate Ca^{2+} concentration resulting in an erroneously small dynamic range between R_{min} and R_{max}.[31] *In vitro* calibration methods were therefore employed in these experiments. However variability in cell autofluorescence, and the inability to determine it for the specific fura-2 loaded cell, led to the underestimation of Ca^{2+} concentrations. Estimated correction for autofluorescence yielded maximal cytosolic Ca^{2+} concentrations from 2 to 5 times reported values.

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FIGURE LEGENDS

FIG. 1. **Repetitive deliveries of 60 mM K^+ to test reproducibility of a single chromaffin cell.** Every 2 min a 3-s application of 60 mM K^+ was given to single chromaffin cells in medium with 2 mM Ca^{2+} as indicated by the arrows. Fluorescence of fura-2 (upper trace) was monitored simultaneously with amperometric current from the oxidation of released catecholamine (lower trace). The vertical axis applies to the fura-2 ratio trace and the scale bar in the bottom right corner quantitates oxidative current of catecholamine release spikes. The inset shows the mean maximal cytosolic free Ca^{2+} concentration (open bars) and release of catecholamine for 1 min following stimulation (solid bars) normalized to the first stimulation as a function of order of stimulation delivery (n = 5 cells).

FIG. 2. **Concentration dependence of peak cytosolic free Ca^{2+} and catecholamine release from stimulation with high K^+.** A 3-s application of various concentrations of K^+ was given every 2 min to a single chromaffin cell as indicated by the arrows. Fura-2 ratio fluorescence (upper trace) was monitored simultaneously with amperometric current from the oxidation of released catecholamine (lower trace).

FIG. 3. **Dose response of peak cytosolic free Ca^{2+} and catecholamine release versus log [K^+].** (A) Plot of peak cytosolic free Ca^{2+} concentration versus log [K^+]. (B) Plot of catecholamine release versus log [K^+]. A linear regression is fit to the portion of the curve where K^+ is sufficient to cause catecholamine release (r = 0.982). In all cases, the results were normalized to those obtained with 140 mM K^+. Each data point is from duplicate stimulations at 6 cells. Error bars are the standard errors of the mean.

FIG. 4. **Concentration dependence of peak cytosolic free Ca^{2+} concentration and catecholamine release from stimulation with DMPP.** A 3-s application of various concentrations of DMPP was given every 2 min to a single chromaffin cell as indicated by the arrows. Fura-2 ratio fluorescence (upper trace) was monitored simultaneously with

amperometric current from the oxidation of released catecholamine (lower trace). The inset shows the peak cytosolic free Ca^{2+} concentration (open bars) and secretion of catecholamine for 1 min following stimulation (solid bars) as a function of DMPP concentration. The bars are the mean ± s.e.m normalized to the mean for the maximal dose (50 μM). Each bar represents data from duplicate stimulations at 6 cells.

FIG. 5. **Cytosolic free Ca^{2+} oscillations and catecholamine release at a single cell due to exposure to veratridine.** A 3-s delivery of 60 mM K^+ was first given to test the viability of the cell before exposure to veratridine. Veratridine was then added to the culture dish to give the indicated concentrations during the times marked by the bars. (Note the artifact created in both traces by activity during drug addition.) Fura-2 fluorescence ratio (upper trace) was monitored simultaneously with release of catecholamine (lower trace). The inset is an expansion of the time period directly following exposure to 100 μM veratridine. Oscillations of cytosolic free Ca^{2+} (upper trace) and catecholamine release (lower trace) can be seen to temporally coincide in this portion of the trace.

FIG. 6. **Cytosolic free Ca^{2+} and catecholamine responses to caffeine in the presence and absence of external Ca^{2+}.** 10 mM and 40 mM caffeine was alternately applied to cells (A) in the presence of 2 mM extracellular Ca^{2+} (n=5) and (B) in medium containing 0.2 mM EGTA (n=7). Fura-2 fluorescence ratio (dashed lines) was monitored simultaneously with release of catecholamine (solid lines). Under both conditions, 10 μM DMPP was delivered for 3 s before and after the caffeine study in order to confirm cell viability. In the experiments in medium containing 0.2 mM EGTA, the DMPP pipette solution also contained 2 mM Ca^{2+} so that fura-2 responses and release could be confirmed. This transient delivery was sufficient to refill the depleted caffeine-sensitive stores in experiments without extracellular Ca^{2+}.

FIG. 7. **The variety of catecholamine release and cytosolic free Ca^{2+} responses to bradykinin in medium containing 0.2 mM EGTA.** A 5-s delivery of 200 nM bradykinin was given to 15 cells in the absence of extracellular Ca^{2+}. (A) Four of the cells studied resulted in an

increase of cytosolic free Ca^{2+}, presumably from IP_3-sensitive internal stores, and resultant exocytosis of catecholamine. (B) One of the cells studied showed a sustained increased in cytosolic free Ca^{2+} but did not cause catecholamine release. The break in the traces indicates a 40 s pause. (C) Ten of the cells did not induce substantial cytosolic free Ca^{2+} rise or exocytotic release. For experiment in media with 2 mM Ca^{2+}, results as in A (n=5), B (n=2), C (n=2) were obtained. Transient applications (3 s) of 60 mM K^+ and 2 mM Ca^{2+} were given before and after the bradykinin study to ensure cell viability.

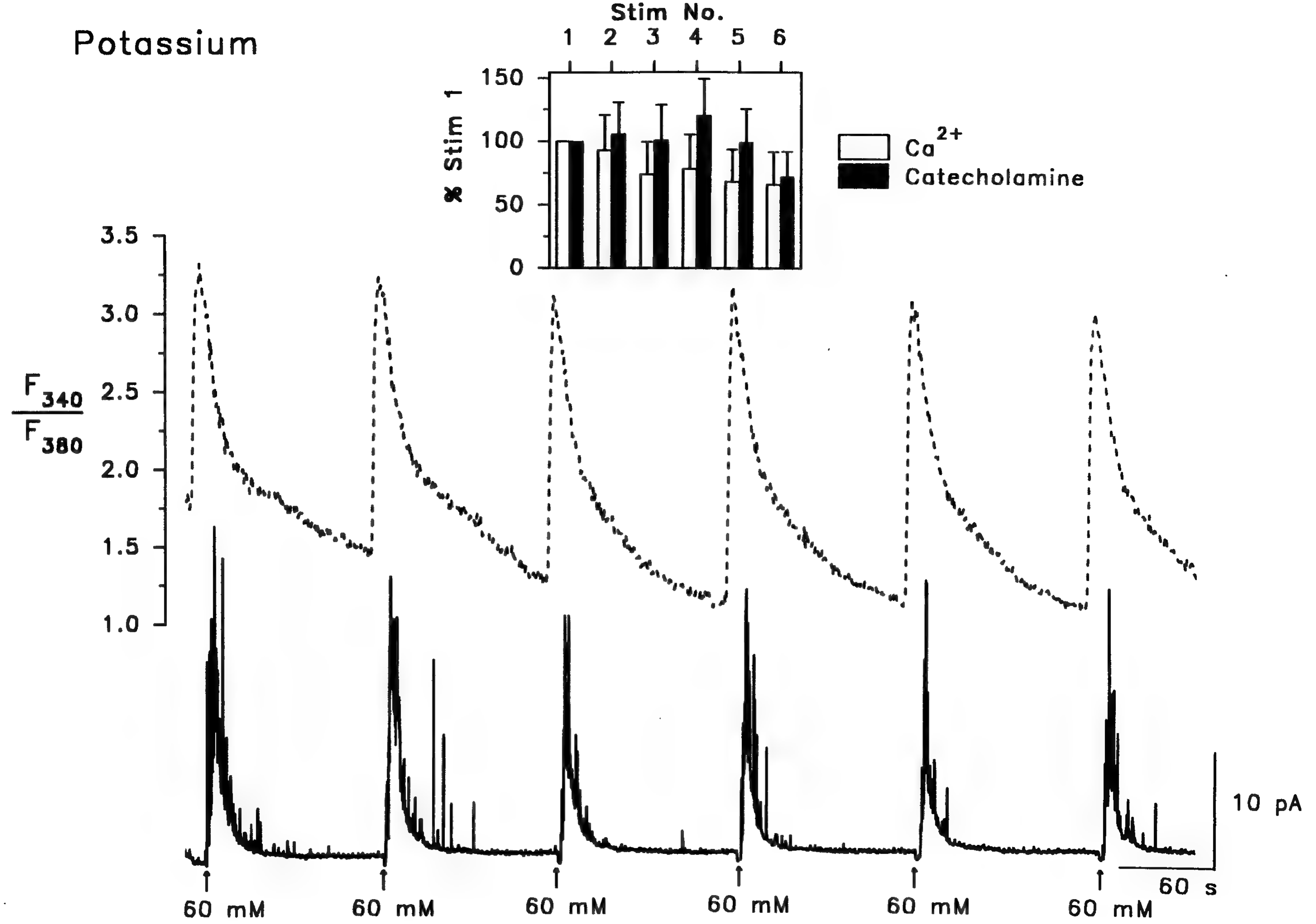
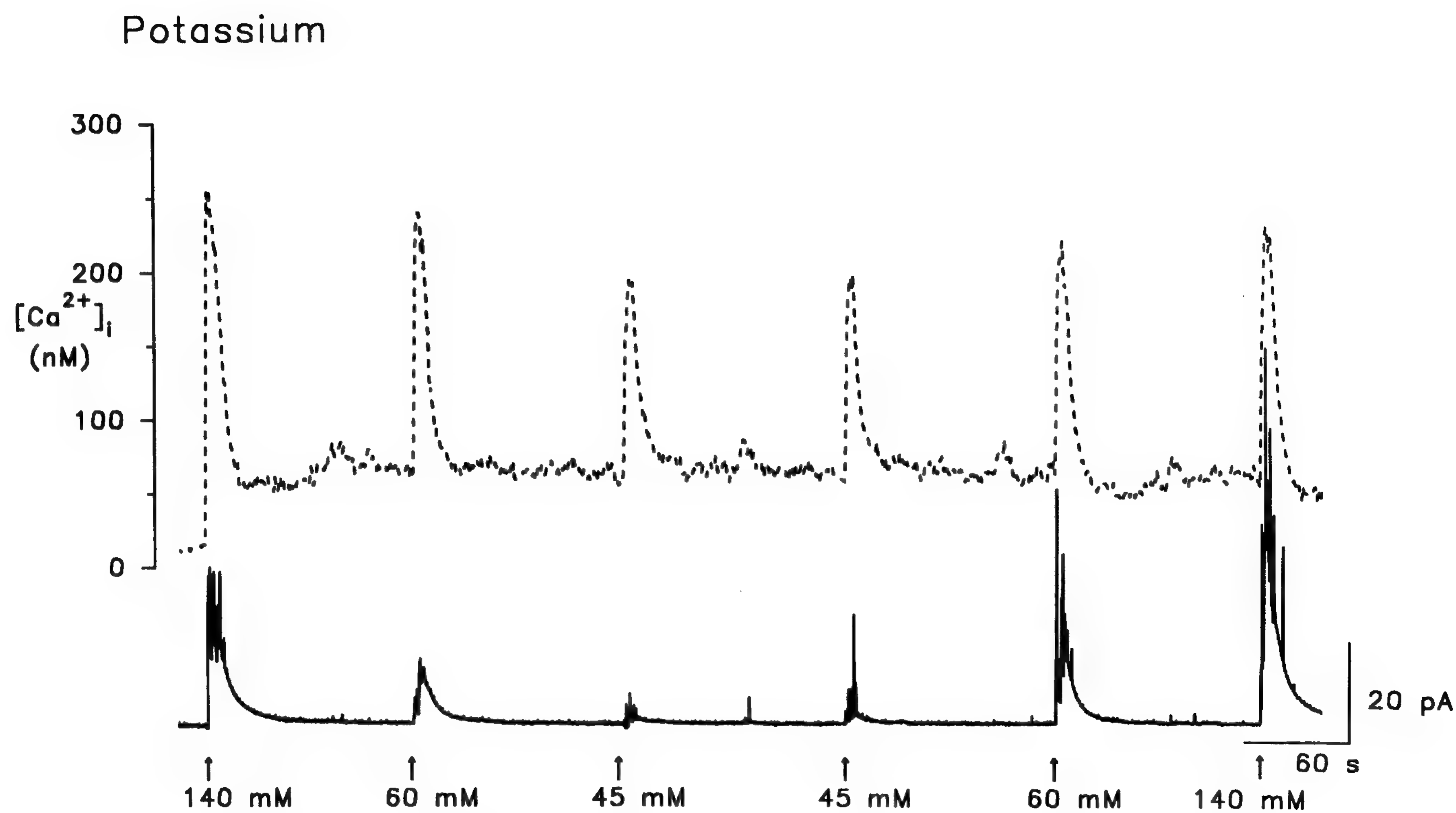

Potassium
Stim No.
1 2 3 4 5 6
150
100
50
0
% Stim 1
Ca2+
Catecholamine
3.5
3.0
2.5
2.0
1.5
1.0
F340/F380
10 pA
60 s
60 mM
60 mM
60 mM
60 mM
60 mM
60 mM

FIGURE 1



Potassium
300
200
100
0
[Ca2+]i
(nM)
20 pA
60 s
140 mM
60 mM
45 mM
45 mM
60 mM
140 mM

FIGURE 2

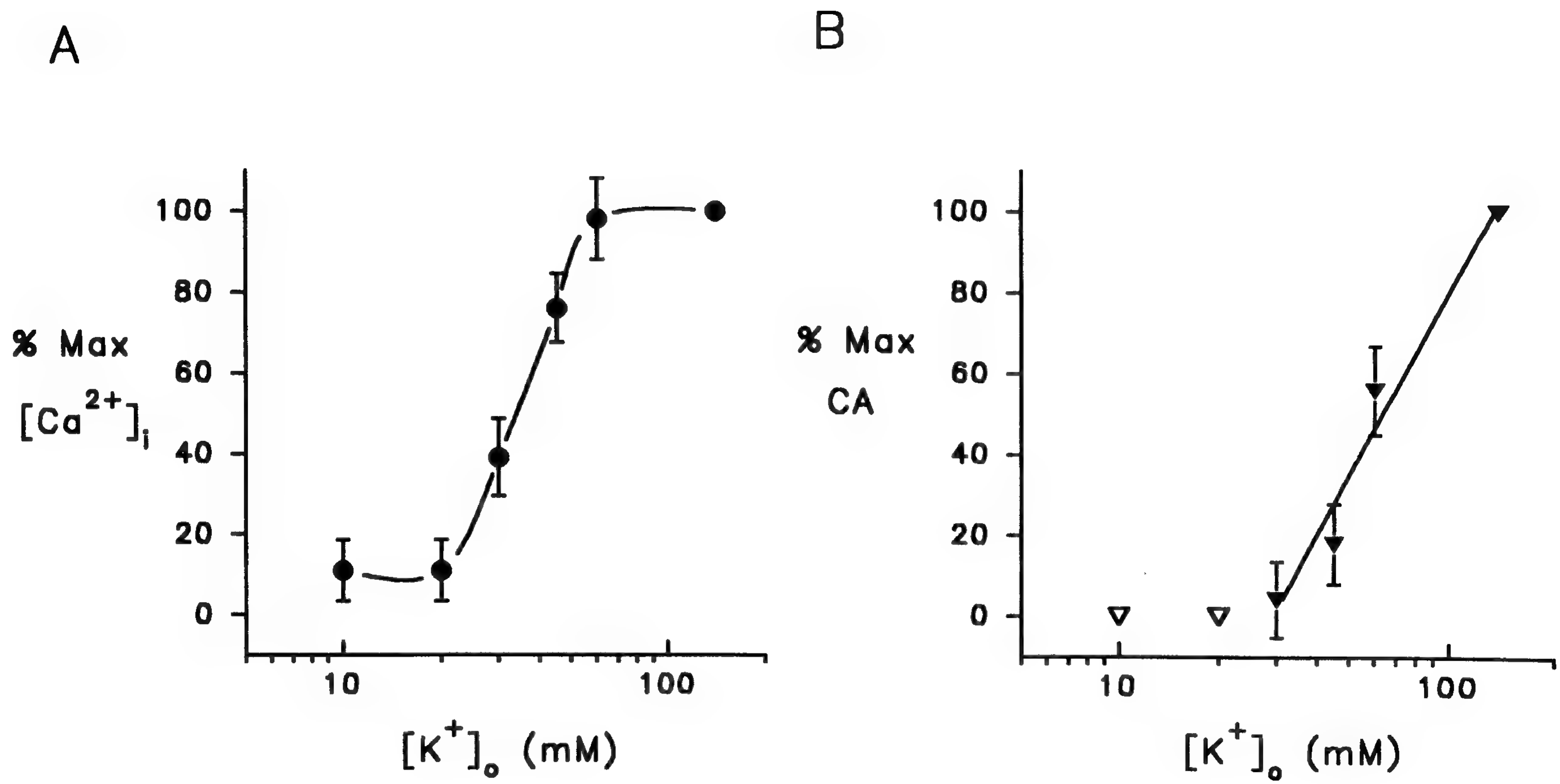


FIGURE 3

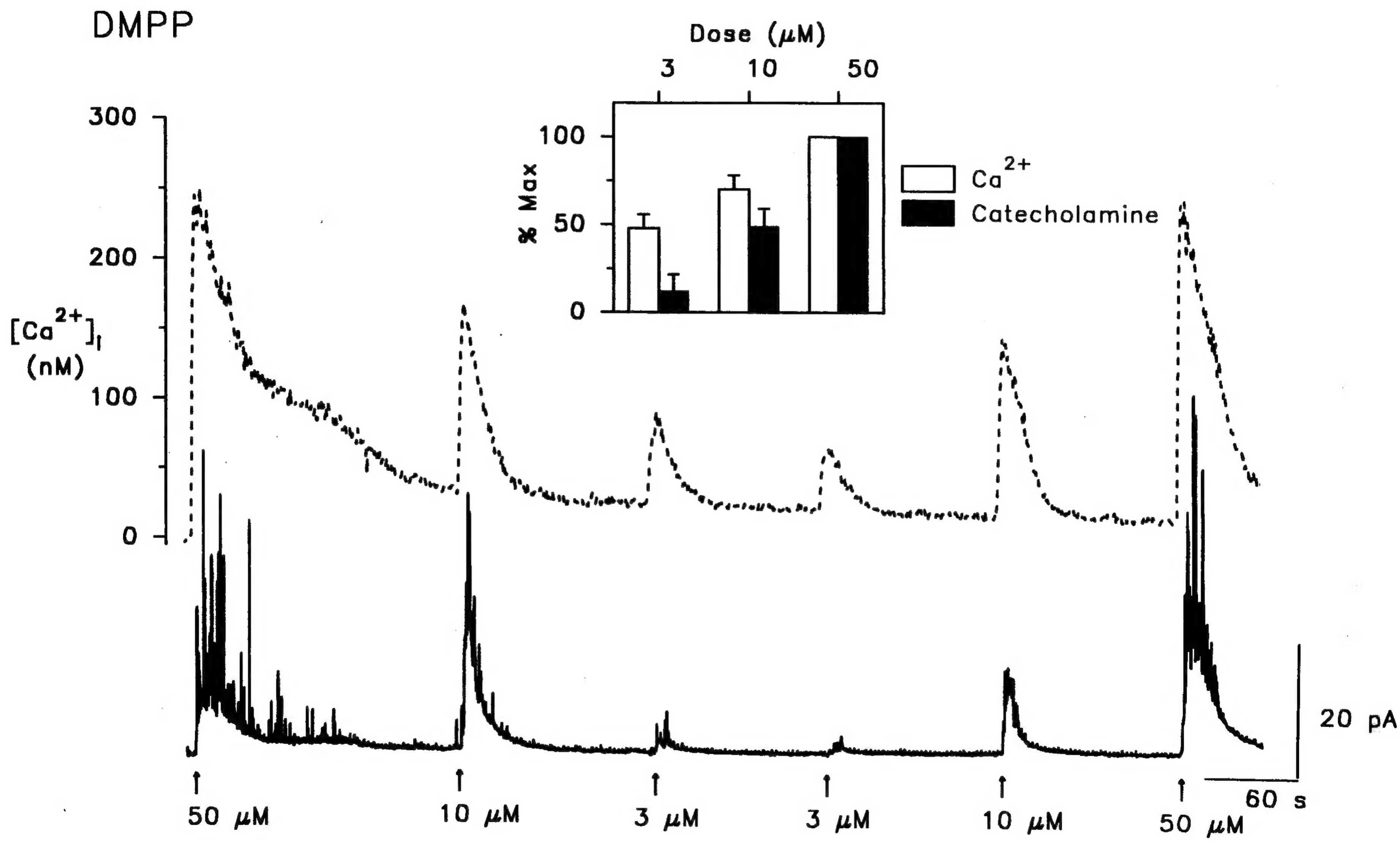

DMPP
Dose (μM)
3
10
50
100
50
0
% Max
Ca2+
Catecholamine
300
200
100
0
[Ca2+]i
(nM)
20 pA
60 s
50 μM
10 μM
3 μM
3 μM
10 μM
50 μM

FIGURE 4

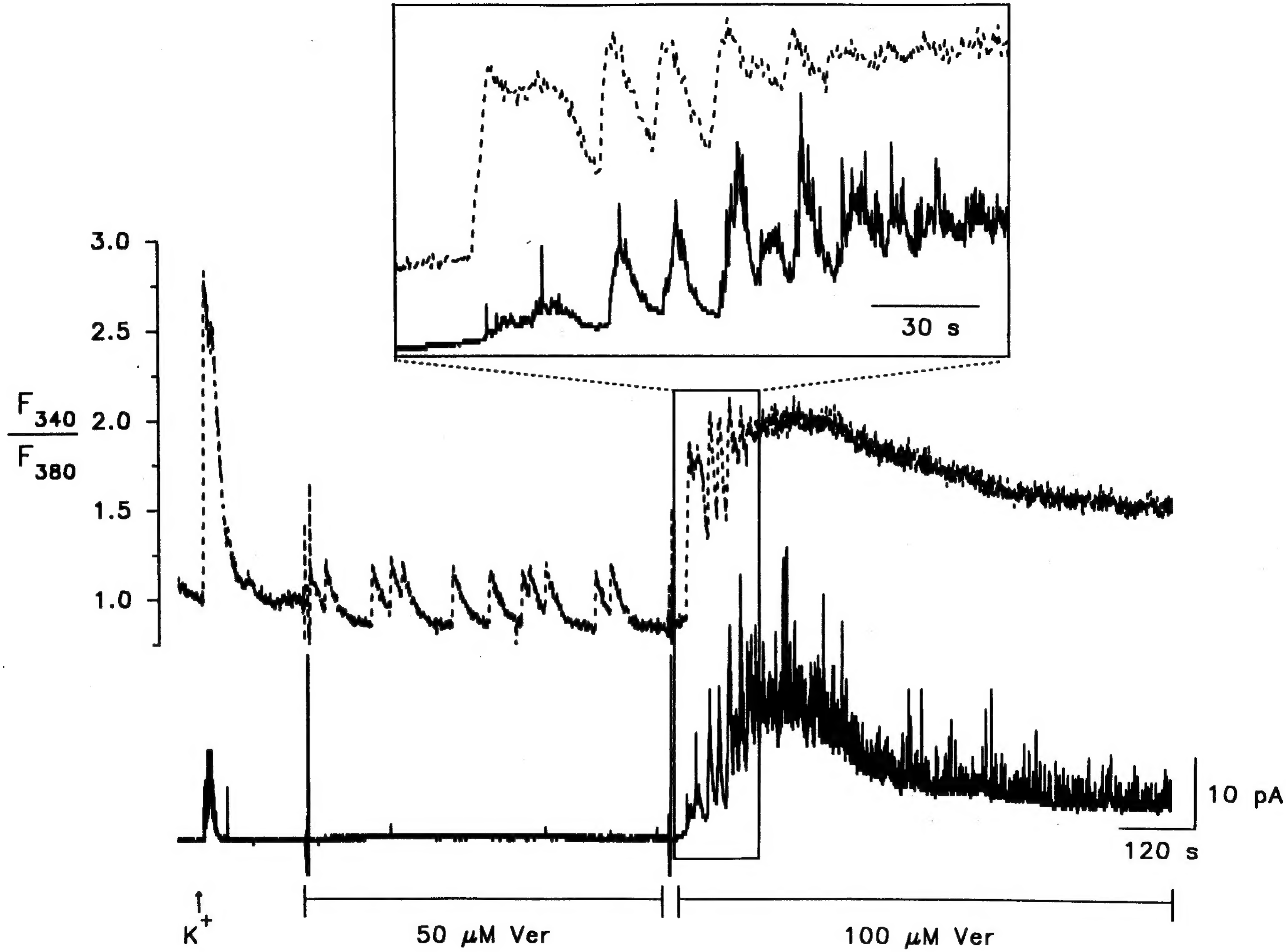


FIGURE 5

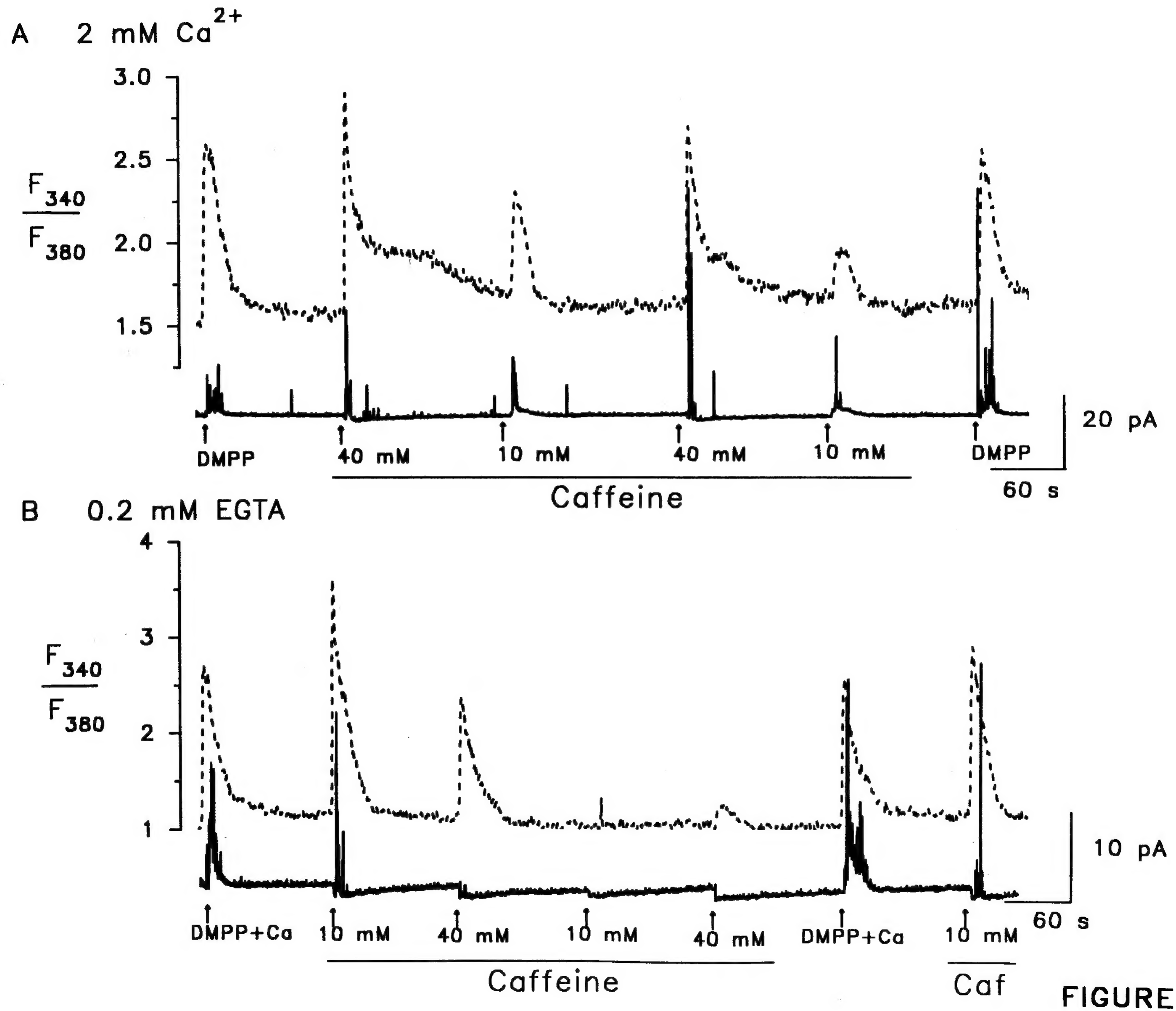

A 2 mM Ca2+
3.0
2.5
2.0
1.5
F340/F380
DMPP
40 mM
10 mM
40 mM
10 mM
DMPP
20 pA
60 s
Caffeine
B 0.2 mM EGTA
4
3
2
1
F340/F380
DMPP+Ca
10 mM
40 mM
10 mM
40 mM
DMPP+Ca
10 mM
10 pA
60 s
Caffeine
Caf

FIGURE 6

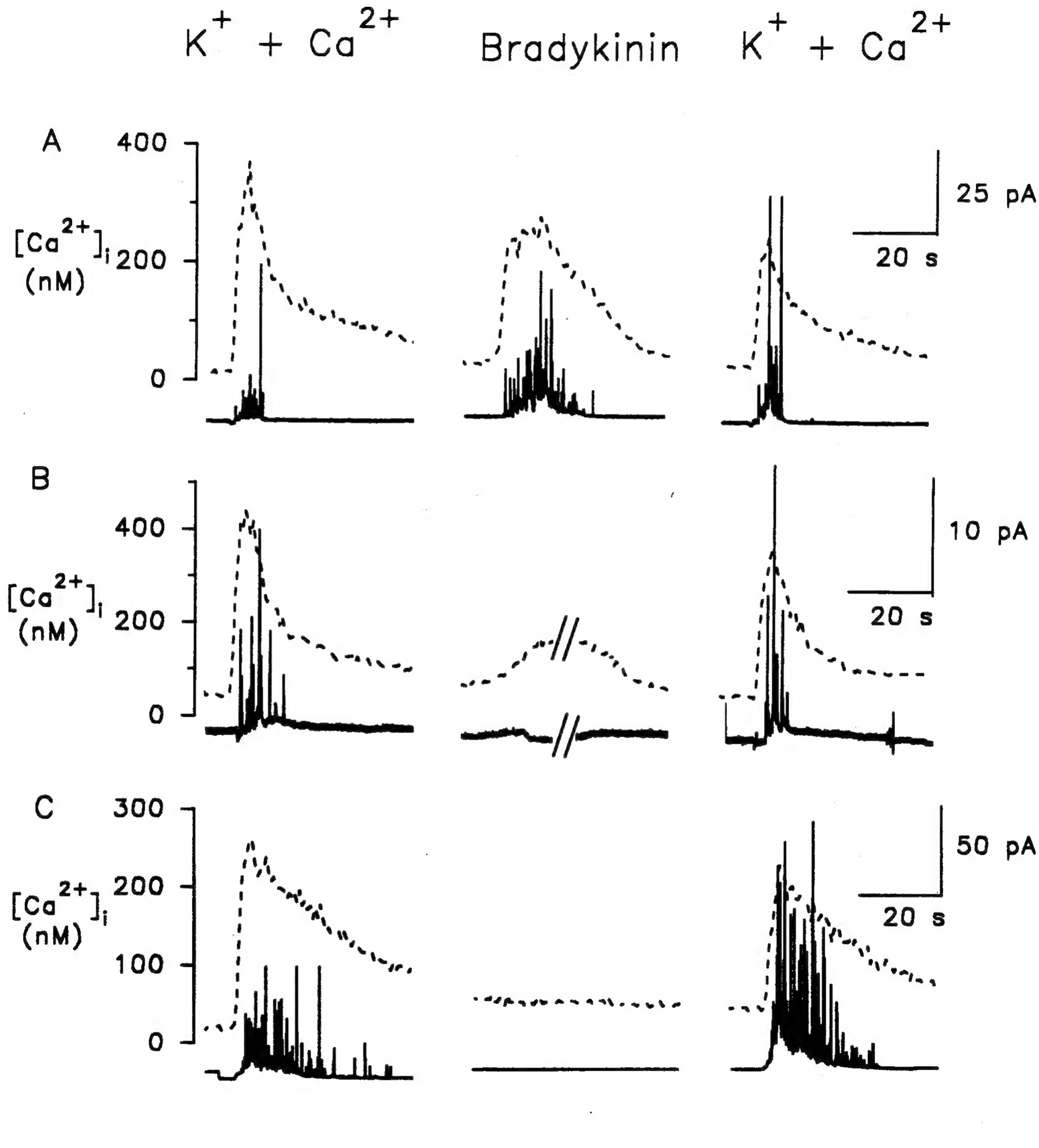

K+ + Ca2+
Bradykinin
K+ + Ca2+
A
400
200
0
[Ca2+]i
(nM)
25 pA
20 s
B
400
200
0
[Ca2+]i
(nM)
10 pA
20 s
C
300
200
100
0
[Ca2+]i
(nM)
50 pA
20 s

FIGURE 7